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Inter-isolate variation for virulence in *Plasmopara halstedii* (sunflower downy mildew) from Hungary

F. VIRÁNYI* *and* T. J. GULYA†
Plant Protection Institute, Hungarian Academy of Sciences, 1525 Budapest, Hungary and †USDA, Northern Crop Science Laboratory, Fargo, North Dakota 58105, USA

A total of 82 isolates of *Plasmopara halstedii*, collected from all production areas of Hungary between 1976 and 1993, were assessed for their virulence pattern on a standard set of sunflower differentials under glasshouse conditions. The isolates were classified into six pathogenic races each representing a particular virulence phenotype. From 1976 until 1988 all the isolates were found to be virulent only on sunflowers possessing no known resistance genes, thus classified as race 1. There was an apparent shift in the virulence of the *P. halstedii* population collected after 1988. Six races (1, 2, 3, 4, 8 and 9) were identified among the 45 samples collected between 1989 and 1993, with races 1 and 3 predominant, at a frequency of 35% each. While the increase in race virulence is undoubtedly due to selection imposed by resistant hybrids, the origin of the new races is unknown. Whether new races have arisen from the indigenous *P. halstedii* population, or whether they have been imported from abroad, can only be reliably determined by DNA techniques, such as fingerprinting.

INTRODUCTION

Plasmopara halstedii (PH), the causal agent of downy mildew of sunflower (*Helianthus annuus*), is a destructive pathogen worldwide. Mildewed sunflower plants do not usually produce viable seed and, once they are systemically infected, they cannot recover from the disease. The fungus is an obligately biotrophic Oomycete which reproduces asexually by means of motile zoospores released from sporangia, and sexually through soilborne oospores. Upon entering the host plant, it colonizes the underground tissues of young seedlings and, by growing upwards, it becomes systemic, resulting in the appearance of various symptoms, the most typical of which are severe stunting and characteristic chlorotic patterns on affected leaves. The fungus sporulates freely on invaded cotyledons and leaves if environmental conditions are favourable but, unlike other downy mildews, wind-blown spores and subsequent localized foliar lesions do not contribute to significant disease spread (Sackston, 1981).

In contrast to the great variation in disease symptoms induced, PH has been thought for a long time to be uniform in its pathogenicity. In the early 1970s, field isolates of *P. halstedii* in North Dakota (USA) and adjacent areas of Canada were found to differ in virulence from those known to occur in Europe. These isolates were designated as the 'Red River' race after the geographical area in which they first appeared (Zimmer, 1974). This pathogenic race, now called race 2, was followed by the appearance of additional races in America as well as in other parts of the world. An account of the pathogenic race appearance has been published by Gulya *et al.* (1991a) and is summarized in Table 1.

In Europe, the PH population appeared to be much more uniform, with no detectable differences in the virulence pattern on sunflower cultivars grown in this area. In 1988, however, French scientists reported a PH isolate capable of infecting resistant sunflowers (Tourvieille *et al.*, 1988). In the following year, a few field collections from Hungary showed virulence on sunflower cultivars resistant to races 1 and 2 (Virányi & Masirevic, 1990). Subsequent investigations revealed the occurrence of new races from Bulgaria, France, Hungary and Italy, as well as from countries in other continents (Garcia & Gulya, 1991; Gulya & Virányi, 1991; Gulya *et al.*, 1991a; Rashid, 1993). As a result, at least nine pathogenic races of PH are known to occur in North America and Europe. In South

*To whom correspondence should be addressed.

Table 1. *Plasmopara halstedii* race evolution between 1972 and 1993

Race designation	First appearance		Distribution	Selected reference
	Year	Location		
1			Worldwide	Sackston, 1981
2	1972	USA	North America	Zimmer & Kinman, 1972
3	1980	USA	N and S America Europe	Carson, 1981 Gulya *et al.*, 1991a Virányi *et al.*, 1992
4	1985	USA	North America, Europe	Gulya & Urs, 1985 Gulya *et al.* 1991a
5	1988	USA	North America	Ljubich *et al.*, 1988
6	1990	Canada	North America, France	Gulya *et al.*, 1991a
7	1990	Argentina	N and S America	Gulya *et al.*, 1991a
8	1990	USA	USA, Hungary	Garcia & Gulya, 1991 Virányi & Gulya, 1993
9	1993	USA, Hungary	USA, Hungary	Virányi & Gulya, 1993

America only three races have been confirmed, while in Africa race 1 is the only confirmed race. Australia is the only sunflower-producing country from which PH is totally absent (Sackston, 1981; Gulya *et al.*, 1991a; Mouzeyar *et al.*, 1991). The time-course of 'race-evolution' of PH is shown in Table 1.

In the present study we have examined the pathogenic diversity of PH in Hungary over a relatively long period of time, between 1976 and 1993.

MATERIALS AND METHODS

Collection and maintenance of isolates

Isolates of *P. halstedii* were obtained over a 17-year period from the main sunflower-growing regions in Hungary (Fig. 1). The samples were collected from commercial sunflower fields, from 'volunteer' plants, or from breeding nurseries and yield trials. In addition, samples were obtained from cooperators' collections in local plant protection stations and other government institutions, whose assistance is gratefully acknowledged. In most instances, sporangia were collected from single downy mildew-infected sunflower plants, but in a few cases, PH was isolated from *Xanthium strumarium*, an alternate host of PH which displays systemic symptoms identical to those of cultivated sunflower (Leppik, 1966; Virányi, 1984).

Sporangia were washed off plants and used to inoculate individual 2- to 3-day-old sunflower seedlings of universal susceptibility (e.g. cvs GK-70 or IS 003) in order to increase and maintain the isolate. Inoculations were made using the whole-seedling immersion (WSI) method (Cohen & Sackston, 1973), with minor modifications (Virányi, 1977; Gulya *et al.*, 1991a, 1991b). A total of 20 to 25 seedlings of each differential line were immersed in a suspension of 20 000–30 000 sporangia ml for 3–4 h at 16–18°C. Inoculated seedlings were then planted in flats containing soil or a sand-perlite mixture (2:1, v/v) and grown in a glasshouse at 22 ± 4°C for 12–14 days with a 16 h photoperiod.

Since PH sporangium preservation techniques were not yet fully developed in the 1970s, most of the earlier isolates were discarded and only a few were maintained by repeated inoculations every 2–3 weeks. Starting in the 1980s, PH sporangia were deep-frozen on cotyledons and stored at −70°C, under which conditions viability was retained for at least 1 year (Virányi, 1985), or sporangia were vacuumed from plants and stored in liquid nitrogen, under which conditions they have remained viable for at least 5 years (Gulya *et al.*, 1993). Most, but not all, isolates collected since 1989 were stored deep-frozen and could thus be re-tested as new differential lines became available.

Differential lines

In the late 1970s and early 1980s, five differentials

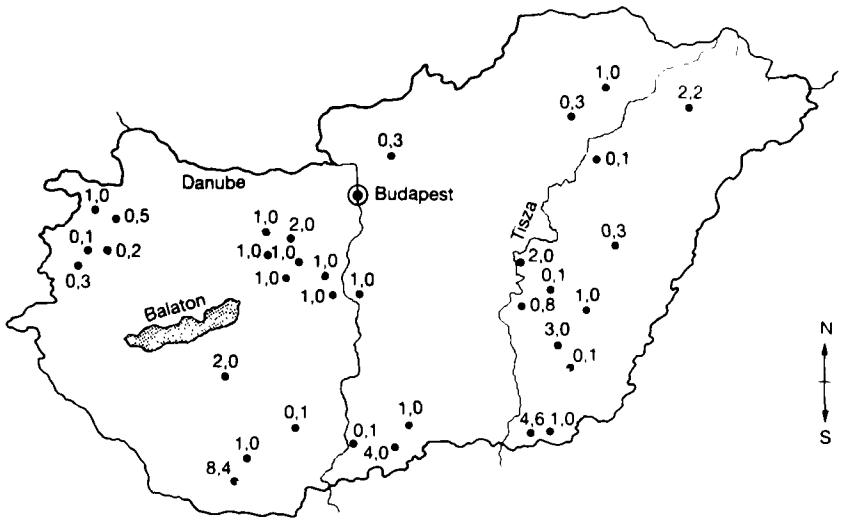

Fig. 1. *Plasmopara halstedii* sampling sites between 1976 and 1993 in Hungary. Black circles indicate the 34 different locations throughout the country where sunflower fields for sampling were selected. Figures preceded/followed by a comma indicate the number of isolations made either during the period 1976–1988, or during the period 1989–1993, respectively.

were commonly used. They included a universal suscept (GK-70 or Iregi szürke csikos), a line with resistance only to race 1 (AD-66 or RHA-265), and the lines HA-61, HIR-34 and RHA-274. The origin, the postulated resistance genes (Pl genes), and the reactions of these and subsequent differentials are listed in Table 2. With the discovery of resistance to new races, and the release of homozygous lines, new differentials became available in the late 1980s (Miller & Gulya, 1987, 1991; Tan *et al.*, 1992). Inheritance studies have been performed on

Table 2. Reaction of sunflower differential lines to known pathogenic races of *Plasmopara halstedii*

Line	Postulated R-gene	Reaction to race								
		1	2	3	4	5	6	7	8	9
GK-70[a], Iregi[a], IS-003[b]	None		+	+		+	+	+	+	+
AD-66[c], RHA-265[d]	Pl_1	–	+	+	+	+	+	+	+	+
HA-61[d]	$Pl_{2,3,+?}$	–	–	+	+	+	–	–	+	–
HIR-34[e]	$Pl_{4,+?}$	–	–	+	+	+	–	–	+	–
RHA-274[d]	$Pl_{2,9,10}$	–	–	+	+	+	–	–	+	–
RHA-325[d]	$Pl_{2,+?}$	–	–	+	+	+	+	–	+	–
DM-2[d]	$Pl_{2,5,+?}$	–	–	–	+	+	+	+	+	+
IS-2000[b]	$Pl_{2,5,+?}$	–	–	–	+	+	–	–		+
803-1[d]	?	–	–	–	–	+	–	–	–	–
HA-335[d]	$Pl_{6,+?}$	–	–	–	–	–	–	–	–	–

[a] Open pollinated cultivars from Hungary
[b] Proprietary line from Interstate (Van der Haave) USA.
[c] Public inbred line from ICCPT, Fundulea, Romania.
[d] Public inbred lines from USDA Oilseeds Unit, Fargo, ND, USA.
[e] Public inbred line from INRA, Clermont-Ferrand, France.
+ Susceptible; –, resistant; s, stunting with no apparent sporulation.
* Question mark denotes the presence of postulated but not verified resistance gene(s).

Table 3. Race character of *Plasmopara halstedii* field isolates collected between 1976 and 1993 in Hungary

Year	Number of isolates	Race								
		1	2	3	4	5	6	7	8	9
1976[a]	1	1		–	–	–	–	–	–	–
1981	6	6	–	–	–	–	–	–	–	–
1982	14	14		–	–	–	–	–	–	–
1983	2	2		–	–			–	–	–
1984	2	2		–	–			–	–	–
1985	8	8	–	–	–		–	–	–	–
1987	1	1	–	–	–			–	–	–
1988	3	3	–	–	–		–	–	–	–
1989	3	–	–	1	1			–	–	1
1990	11	4		4	1			–	1	1
1991	18	4	2	8	2	–		–	1	1
1992	8	7	–		–			–	–	1
1993	5	1	–	2	2	–		–	–	
Total	82	53	2	15	6	–	–	–	2	4

[a] Four samples of *P. halstedii* were collected from different locations in Hungary in 1976, mixed together and subsequently regarded as a single isolate, designated H-1.

some of these lines (Vear & Leclercq, 1971; Zimmer & Kinman, 1972; Zimmer, 1974; Vranceanu *et al.*, 1981; Miller & Gulya, 1987, 1991; Tan *et al.*, 1992). These lines have been tested repeatedly and found to react consistently and uniformly with all pathogenic races to date (Gulya *et al.*, 1991b).

Race identification

For race identification, each isolate was inoculated on to a universal suscept in order to produce fresh inoculum. Fresh sporangia were used to WSI-inoculate 20–25 seedlings of each differential line, which were then grown as described above. After 12–14 days in the glasshouse, at which time the first true leaves on most differentials were 2–3 cm long, the seedlings were transferred to a humid chamber at 18°C overnight in order to induce sporulation. Susceptibility was interpreted on the basis of the presence of sporulation on cotyledons and/or on true leaves. A test was considered satisfactory only if the universal suscept showed infection of 95–100% of plants. All tests were repeated at least twice. Most isolates collected during the 17-year period produced a high (90–100%) level of infection on both the universal suscept and, in cases of compatibility, on other lines. If a differential had a much lower percentage of infected plants, an additional test was performed. If the results were similar, we interpreted this as being due to a mixture of races. Further details of the methodology have already been reported by Gulya *et al.*(1991a, 1991b), and some preliminary results have already been published (Gulya *et al.*, 1991a; Virányi *et al.*, 1992; Virányi & Gulya, 1993).

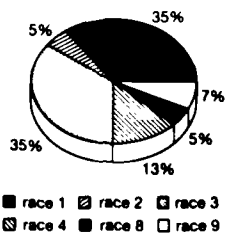

Fig. 2. Race composition of 45 sunflower downy mildew isolates collected between 1989 and 1993 in Hungary. Different black-and-white patterns represent the relative occurrence of fungal races expressed as a percentage of the total during the time period studied.

RESULTS

A total of 82 PH isolates were collected and identified over the 17-year period. In some years

and in some regions the weather during the spring was unfavourable for mildew infection, and as a result fewer samples were collected. Samples were obtained fairly uniformly from several different climatic and geographical regions of Hungary, including the Hungarian Great Plain along the Tisza river, the South-Central and the Northern territories, as well as the so-called Trans-Danubian area (west of the river Danube) (see Fig. 1). While the number of samples collected in any given year varied considerably (Table 3), an approximately similar number was collected between 1976 and 1988 (37 isolates but 40 samples, isolate H-1 being a mixture of four samples collected in different locations in 1976), and between 1989 and 1993 (45 isolates).

Between 1976 and 1988 a total of 37 isolates were examined, all of which were classified as race 1, i.e. they were unable to infect any differentials other than the universal suscept (Table 3).

However, for the period between 1989 and 1993 a totally different picture of race diversity emerged. From the total of 45 isolates examined, six races were identified, including races 1, 2, 3, 4, 8 and 9 (Table 3). Among these, race 2 was new to Hungary and race 9 was new to Europe. Figure 2 shows the relative ratio of PH races identified between 1989 and 1993. The percentage data for race incidence suggest that there was a substantial shift in the Hungarian PH population after 1988. The absolute dominance of race 1 prior to 1989 changed, and races 1 and 3 became co-dominant, with at least four additional races present at low levels.

DISCUSSION

The sampling intensity from the different regions of Hungary is not sufficient to allow any assessment of the presence/absence of races by region, or of the dominance of any race other than races 1 and 3 in all areas after 1988. However, the data for racial diversity of PH in Hungary suggest that the fungal population has changed considerably from only one race to at least six races.

Although all six races identified are also present in North America (Gulya *et al.*, 1991a; Rashid, 1993), no valid conclusions can be drawn regarding the origin of the 'new' races in Hungary. While they may have been imported via seed from North America, they could also have arisen from the indigenous Hungarian or European PH populations. Since sunflower hybrids grown in North America and Europe mainly possess resistance to races 1 and 2, as well as to races 6, 7 and 9 (Gulya, unpublished data), the increase in more virulent races is not surprising. If it were not for the presence of non-resistant 'volunteer' plants or wild *Helianthus* species, the incidence of the less virulent races might have decreased even sooner.

The uniformly high level of infection observed in all differentials in compatible combinations indicates that sporangia collected from individual plants represented single races. In inoculum dosage experiments, we have observed approximately 5% infection with a sporangial concentration of only 200 spores/ml. Thus, within our test parameters of a standardized inoculum of 20 000/ml and a minimum of 20 seedlings/line, we are able to detect race mixtures as low as 1% (200 out of 20 000) when only one seedling of a line is infected (assuming the universal suscept is totally infected) (Gulya, 1991). In our opinion, had we chosen to make single-spore cultures of all isolates, this could have falsely altered the racial diversity that we observed. Since single-sporangial cultures are quite time-consuming (approximate efficiency 10%), racial diversity within individual fields and within individual plants will be the subject of a separate study.

The existence of at least six pathogenic races of PH in Hungary, coupled with the fact that no resistance genes against the well-distributed races 3, 4 and 8 are available in the commercially grown sunflower hybrids to date, emphasize the importance of a permanent programme for monitoring of the pathogen population. Such a programme would provide information needed by both breeders and growers in order for them to make optimal decisions regarding the control of this devastating disease.

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